AGROBACTERIUM TUMEFACIENS CROSS-REACTING ANTIGENS IN STERILE CROWN-GALL TUMORS

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1. Introduction

In the past decade remarkable progress has been made in tumor immunology. Several reviews have been published on chemically induced animal tumors [1], tumors induced by DNA viruses (polyoma, SV40, adenovirus [2]) and tumors and leukemias induced by RNA viruses (avian and murine leukemia sarcoma viruses, mammary tumor virus [3]).

Virus free tumors and transformed cells, induced by DNA viruses synthesize two types of virus specific antigens, distinct from viral structural proteins. One type is called the transplantation antigen and is located on the cell surface. For the other type the name neoantigens or T.antigens is used.

In lytically infected cells the T. antigens appear early, before viral structural proteins. The neoantigens are soluble, in part heat labile and located in the nucleus of the host cell. Their formation is regarded as evidence for the presence of parts of the viral genome in the transformed cells. This conclusion is supported by the results of recent investigations in two different ways. One of them is the so-called hybrid induction, in which fusion of the transformed cell with virus susceptible cells, gives rise to the production of infective virus [4]. The second is the presence in transformed cells of RNA complementary to DNA of the transformation inducing viruses [5, 6].

A few years ago we reported [7] evidence for the presence of genetic material of the inducing bacterial organism in Crown-gall tissue, not present in normal tissue. In the same paper we stated that RNA complementary to DNA of the highly tumorgenic strain B_6 can also be hybridized to DNA from tumors induced by strain A_6. From these results the conclusion could be drawn that, in regard to the appearance of genetic material in the tumor, both tumorogenic strains are related.

More evidence, supporting our findings, can be found in recent papers. Quétier et al. [8] found complementarity between *A. tumefaciens* DNA and Crown-gall tissue DNA. Milo et al. [9] detected RNA in Crown-gall tissue complementary to *A. tumefaciens* DNA. They also confirmed that, especially with respect to the bacterial genetic material in tumor tissue, different tumorogenic strains may be related. Moreover it proved possible to transform *in vitro* normal plant tissue into auxin-prototrophic tissue with large amounts of *A. tumefaciens* DNA [10, 11].

To our knowledge no data are available until now about the presence of *A. tumefaciens* antigens in bacteria free Crown-gall tumors. In Crown-gall tumor tissue 20% of the protein content seems to be a "foreign" protein not detectable in normal and habituated tissue [12]. Braun reported a slight but apparently specific precipitation reaction for an antigen present in Crown-gall tumor tissue and absent in normal tissue of the same plant species (parthenocissus) [13]. However, he could not detect the same antigen in Crown-gall tissue from distantly related plants.

Another difference between tumor and normal tissue is the presence of a qualitatively different RNase in tumor tissue. Habituated tissue and *A. tumefaciens* extracts lack this enzyme [14].

The present paper reports the presence of *A. tume-*

faciens cross-reacting antigens in sterile Crown-gall tumors of tobacco, var. White burley, induced by strain A_6. These antigens could not be found in normal tissue of tobacco.

2. Materials and methods

Tumor and normal tissue have been grown *in vitro* for about 6 years and cultivated and tested for sterility as described earlier [7].

Tissue extracts were made by disruption of the tissue in Sørensen buffer with a French pressure cell (Am. Instrum. Co. Inc.) at 10 000 kg/cm^2, followed by ultrasonic treatment at 16–24 kcycle/s for 5 min using a Mullard ultrasonic power unit (60 W).

The soluble antigen preparation was isolated by centrifugation, successively at 3000 *g* for 20 min and 100 000 *g* for 3 hr. The solutions were concentrated by dialysis against polyethylene glycol 6000 till the final volume was about 1/10 of the original tissue weight (taking 1 g tissue = 1 ml). The concentrated solution was dialyzed against 0.0125 M K_2HPO_4 + 0.0125 M KH_2PO_4; pH 7.4 for several hours.

The bacterial antigens were made from *A. tumefaciens* strains A_6, B_6, E III 9.61 and *A. radiobacter.*

One day old cultures grown in synthetic medium were used. The bacteria were harvested by centrifugation, washed (3 ×) with saline, whereupon 1 vol. packed cells was suspended in 1 vol. 0.15 M NaCl – 0.01 M phosphate buffer pH 7.4. The suspension was shaken with 1 vol. of glass beads (diam. 0.17 mm) for 3 min in a Braun MSK homogenizer. Further treatments were the same as for the tissue extracts.

Rabbit antisera to the tissue antigens were obtained by intramuscular injections (at least 6 ×) with sonicated homogenates and concentrated soluble antigens (3 ×), all including adjuvants.

The bacterial antisera were obtained by two intraperitoneal injections with heat killed bacteria (60°C, 45 min), followed by intramuscular injections of viable bacteria and bacterial soluble antigens (with adjuvants).

Blood was collected about two weeks after the last injection.

Ouchterlony immunodiffusion tests were performed according to the method of Crowle [15]. We used 1% Noble agar (Difco) in 0.025 M phosphate – 0.0025 M EDTA buffer, pH 7.4.

The slides were incubated at 4°C. As optimal precipitation for the bacterial as well as the plant tissue antigens with their homologous antisera occurred after 7 days, this incubation time was used for all of our tests.

3. Results and discussion

From fig. 1a, b it is apparent that there are four precipitation lines formed when A_6 soluble antigens are tested against tumor tissue antiserum. These reactions are found with the antisera of both rabbits, although one gave better results than the other. The best antiserum was used for all subsequent experiments.

The cross-reactive antigens were detected in all of the antigen preparations of the virulent strains when tested against tumor antiserum.

A. radiobacter antigens gave precipitates too, but there was a difference in the number of bands with respect to the virulent strains. This avirulent *Agrobacterium* gave 2 lines, while the virulent strains gave 4 lines as can be seen from fig. 2a, b. They have two antigens in common.

These cross-reactions are not found when bacterial antigens are tested against both normal tissue antisera or against sera obtained from rabbits before the first injection with antigens.

The cross-reacting antibodies can be removed from antitumor serum by absorption with the bacterial antigens, but not with normal tissue antigens. The tumor specific antigens are not endotoxines of the tumor inducing bacteria.

The endotoxines (Boivin antigens) were extracted with TCA (5% TCA, 24 hours at 4°C). The extracts gave an excellent reaction with the homologous bacterial antisera, but not with the antitumor sera. The bacterial antigens corss-reacting with antitumor serum were sensitive to heat treatment (60 min, 100°C), in contrast with the bacterial endotoxines, and they were digested by pronase.

Bacteria-free, concentrated media from 1 up to 4 day old bacterial cultures were tested against antitumor serum. No precipitation lines could be detected. This indicates that the tumor specific antigens are not secreted by the bacteria into the culture medium, even after prolonged cultivation. When concentrated

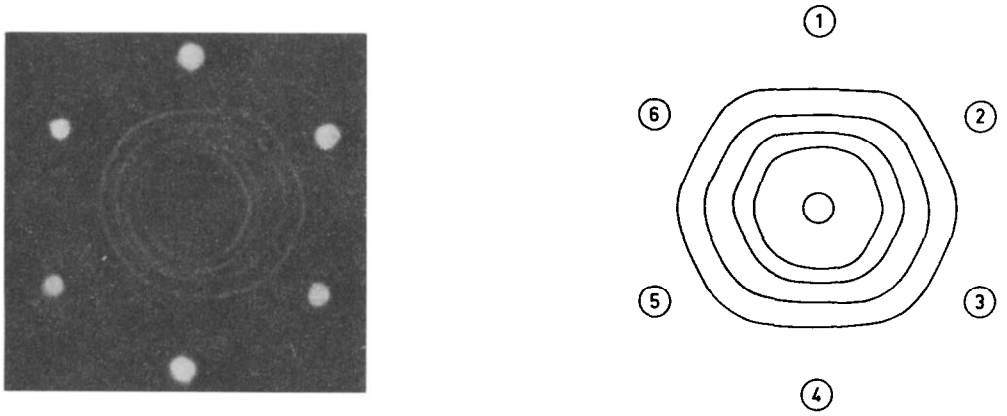

Fig. 1. (a).Microdouble diffusion test of Crown-gall tumor antiserum in the central well and the soluble antigens of the corresponding bacterial strain A_6 in the surrounding wells. (b) Diagramatic representation of 1a.

tumor tissue antigens were allowed to react with A_6 antiserum we did find a number of very faint but distinct precipitation lines. From this weak reaction it is apparent, that the concentration of the "tumor-specific" antigens in our preparations is low. Normal tissue antigens gave no reaction at all.

The existence of these tumor-specific antigens may be part of the expression of bacterial DNA in Crown-gall tumors. The relationship of the different tumorogenic strains with respect to the bacterial genetic material in tumor tissue is reflected in the reaction of their antigens with antiserum of tumor tissue induced by one of them. Of special interest is the different number of precipitation bands found for the non-tumorogenic *A. radiobacter.*

The recent data of Milo et al. are in parallel with our results concerning the tumor specific antigens. They found less, but specific hybridization between tumor RNA and the DNA of a non-pathogenic strain. In our experiments *A. radiobacter* had two antigens in common with the virulent strain A_6, when tested against the A_6 tumor antiserum. The great similarity between DNA from *A. tumefaciens* and *A. radiobacter,* respectively, has been reported previously by Tinbergen [16] and Heberlein et al. [17].

Research on the significance of these antigens in

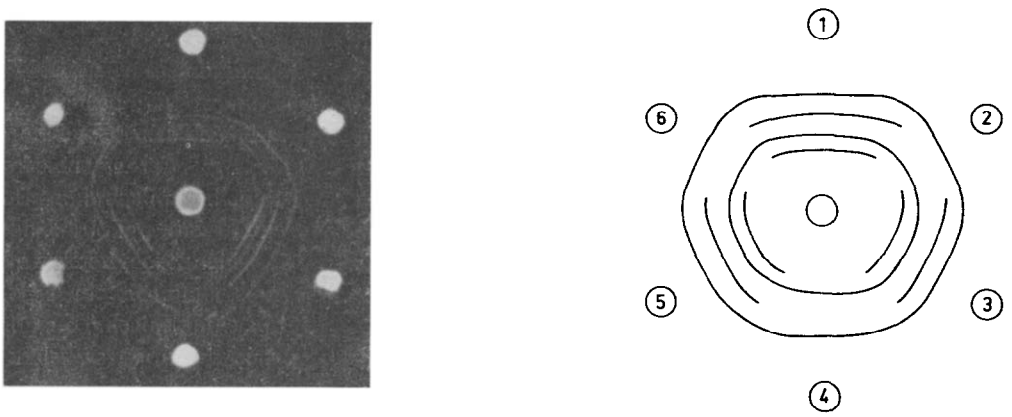

Fig. 2. (a) Microdouble diffusion test of Crown-gall tumor antiserum in the central well and the bacterial soluble antigens of strain A_6 [1, 3, 5] and *A. radiobacter* in the surrounding wells [2, 4, 6]. (b) Diagramatic representation of 2a.

tumor tissue and the difference in cross-reaction with tumor antiserum, between *A. radiobacter* antigens and those of the virulent strains, is in progress.

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